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Automated Analysis of Levosimendan in Human Plasma by On-Line Dialysis and Liquid Chromatography

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A fully automated method for quantitation of levosimendan, (*R*)-(−)-[[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono]propanedinitrile, in human plasma is described. The method involves on-line dialysis of the samples, trace enrichment of the dialysates, and reversed-phase high-performance liquid chromatography with UV detection at 380 nm. An internal standard was used to compensate for variations in the dialysis rate caused by temperature fluctuations. The precision and accuracy of the method were good. The between-day variation (RSD) was 2.7% at a plasma concentration of 15 ng/mL and 1.7% at 450 ng/mL. The limit of quantitation was 5 ng/mL with an RSD of 4.0%. The completion time of the assay was 19 min.

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INTRODUCTION

Levosimendan, (*R*)-(−)-[[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono]propanedinitrile (Fig. 1), is a newly developed calcium sensitizer aimed for the treatment of congestive heart failure (Haikala *et al.*, 1992; Haikala and Lindén, 1995). Levosimendan is the pharmacologically active enantiomer of the racemate simendan. It acts by binding, in a calcium concentration-dependent manner, to troponin-C, the calcium binding protein that regulates contraction of skeletal and cardiac muscles (Haikala *et al.*, 1995; Pollesello *et al.*, 1994).

In early stage pharmacokinetic studies, a manual sample preparation method including laborious liquid–liquid extraction, evaporation, and reconstitution prior to high-performance liquid chromatography (HPLC) was used to determine levosimendan in human plasma. In later studies with a great number of samples an automated method for quantification was needed. One of the most attractive automated sample preparation methods for protein-containing samples is on-line dialysis in combination with enrichment of the dialysate (Turnell and Cooper, 1987; Cooper *et al.*, 1988). Levosimendan is extensively bound to proteins in human plasma. Only the unbound analyte fraction can diffuse through a dialysis membrane, which makes it a challenge to achieve an analyte recovery high enough to provide the sensitivity required of the method (van de Merbel *et al.*, 1992; Herraéz-Hernández *et al.*, 1995).

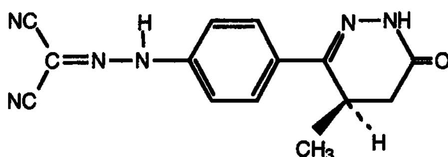

Figure 1. Chemical structure of levosimendan.

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This paper describes a fully automated method for analysis of levosimendan in human plasma. It is based on the ASTED™ system (Automated Sequential Trace Enrichment of Dialysate), connected on-line with an HPLC system. The ASTED system has previously been utilized in several automated methods for analysis of drugs in biological material, in combination with both HPLC and gas chromatography (Agasøster and Rasmussen, 1991a,b; Andresen *et al.*, 1992, 1993; Krogh and Christophersen, 1993; Johansen *et al.*, 1995). Mixtures of chiral compounds present a special problem when dynamic dialysis is used for sample preparation since protein binding, and thus the dialysis rate, is often different for the enantiomers (Williams and Lee, 1985). In the present method, however, dialysis and subsequent achiral chromatography was adequate for specific and accurate determination of the enantiomeric analyte, levosimendan, because it has been demonstrated not to isomerize *in vivo* (Wikberg *et al.*, 1996).

EXPERIMENTAL

Chemicals and reagents. Levosimendan was synthesized by Fermion (Espoo, Finland) and the internal standard, OR-1097, [[4-(1,4,5,6-tetrahydro-6-oxo-3-pyridazinyl)phenyl]-hydrazono]propanedinitrile, by the Department of Synthetic Chemistry of Orion Research (Espoo, Finland). Acetic acid and methanol (HPLC grade) were obtained from Orion-Farmos (Espoo, Finland). Ammonium acetate, sodium chloride (extra pure), sodium dihydrogen phosphate monohydrate, and Triton X-100 were purchased from Merck (Darmstadt, Germany), and tetrahydrofuran (HPLC grade) from Rathburn (Walkerburn, UK). Human serum albumin (HSA, fraction V, 96–99% pure) was from Sigma (St. Louis, MO, USA). Water was purified with a Milli-Q system (Millipore, Milford, MA, USA).

Instrumentation and experimental conditions. The apparatus comprised a Gilson ASTED system (Gilson Medical Electronics,

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Villiers-le-Bel, France) connected on-line to an HPLC system.

ASTED. The ASTED system consisted of a sample injector (Model 231), two dilutors (Model 401) equipped with 1 mL syringes, and a dialysis cell with a donor channel volume of 370 μL. The dialysis cell was fitted with a cellulose membrane (Cuprophan) with a molecular mass cut-off of 15 kDa. A Model 7010 automated six-port valve (Rheodyne, Berkeley, CA, USA) connected a trace enrichment column (10 μm Hypersil ODS, 5.8 × 4.6 mm i.d.) to the recipient channel (650 μL) of the dialysis cell or, after switching, to the HPLC system. The priming solution in the donor side of the system was 0.05% Triton X-100 in a 0.86% sodium chloride solution. The recipient solution was 40 mM ammonium acetate buffer, pH 4.0.

ASTED sample preparation. Through the donor side dilutor, 500 μL plasma was mixed with 25 μL internal standard solution (1.5 μg/mL in 50 mM phosphate buffer, pH 7.2). The donor channel of the dialysis cell was overfilled with this mixture. There it was kept static for 6.6 min while 18 mL recipient solution was pumped through the recipient channel and into the trace enrichment column by the recipient side dilutor. Subsequently levosimendan and the internal standard were eluted from the enrichment column onto the analytical column by back-flushing with HPLC mobile phase. At the same time, residual sample and dialysate were purged out of the dialysis cell by the two dilutors with 10 mL donor solution and 4 mL recipient solution, respectively. Following elution the trace enrichment column was regenerated with 1 mL recipient solution. The overall analysis time was about 19 min per sample.

HPLC. The chromatographic system consisted of an LKB Model 2150 pump (Bromma, Sweden) and a LiChrosorb RP-18 (250 × 4 mm i.d., 10 μm) analytical column (Merck, Darmstadt, Germany). The detector was a Spectra 100 UV-VIS (Spectra-Physics, San Jose, CA, USA). The wavelength was 380 nm and the range 0.01 (Aufs). The mobile phase consisted of a 32 mM monosodium dihydrogen phosphate buffer, methanol, and tetrahydrofuran (45 : 65 : 1, v/v/v). The apparent pH of the mixture was adjusted to 3.5 with phosphoric acid. The mobile phase flow-rate was 1.0 mL/min.

Standards and calibration curves. Plasma standards in the concentration range 5–500 ng/mL were prepared every fourth week by adding appropriate amounts of levosimendan, dissolved in 50 mM phosphate buffer, pH 7.2, to analyte-free EDTA plasma from healthy donors. After careful mixing the plasma was divided in aliquots of about 550 μL and stored in autosampler vials at −20 °C until used. The integrity of plasma was maintained since the volume added was less than 2% of the total volume of the sample. Calibration curves were calculated using linear regression of the peak-height ratio (levosimendan/internal standard) as a function of the levosimendan concentration. The calibration curves ranged from 5 to 500 ng/mL and the method was calibrated for each batch of samples.

Determination of protein binding. To study the plasma protein binding of levosimendan, human plasma was spiked to contain 1.4–140 μg/mL of it. Additionally, a 4.5% solution of human serum albumin (HSA) in 33 mM phosphate buffer, pH 7.4, was spiked to contain 56 μg/mL of levosimendan. Unbound and protein bound levosimendan were separated by ultrafiltration using Ultrafree-MC 10000 NMWL filter units (Millipore, Bedford, MA, USA). Aliquots (400 μL) of samples were pipetted into the filter units and then transferred into the centrifuge in an incubator, the temperature of which was 37 °C. After 30 min the centrifugation (about 1200 *g* at the level of the membrane) was started. The centrifugation time was 30 min. The levosimendan concentrations of the ultrafiltrates were determined with an HPLC method analogous to the one described in this paper after direct injection of 40 μL of the ultrafiltrate. Solutions of levosimendan in 50 mM phosphate buffer, pH 7.4, were used for calibration.

Table 1. Unbound fraction of levosimendan in human plasma and in an HSA solution

Concentration of levosimendan (μg/mL)	Concentration of unbound levosimendan[a] (μg/mL)	Unbound (%)
1.4 (human plasma)	0.03	2.1
14 (human plasma)	0.34	2.4
56 (human plasma)	1.62	2.9
140 (human plasma)	6.08	4.3
56 (4.5% HSA solution)	0.84	1.5

[a] Mean of duplicate determinations.

RESULTS AND DISCUSSION

Protein binding

The relative amounts of levosimendan bound by the protein in plasma and in the HSA solution at various concentations are given in Table 1. The results show that the percentage of unbound levosimendan at concentrations encountered in samples from human studies is about 2%, and that HSA obviously is the protein mainly responsible for binding of levosimendan.

On-line dialysis

Recovery. Only molecules that are not bound to proteins can diffuse through the dialysis membrane. Since about 98% of levosimendan is bound to plasma proteins, attempts were made to reduce the degree of protein binding to facilitate the dialysis process. Addition of reagents known to release drugs from their protein binding sites was tried (Cooper *et al.*, 1988; Agasøster and Rasmussen, 1991b). The reagents investigated were 0.1 M EDTA, 50 mM monochloroacetic acid, buffered trichloroacetic acid (0.2 M, pH 7.2), and 30 mM octanesulphonic acid, but none of these had the desired effect on dialysis rate. However, during experiments with different recipient solutions it was noticed that the pH of the recipient had a pronounced effect on the recovery of levosimendan from plasma. A gradual change in the pH of the recipient (40 mM ammonium acetate) from 6.8 to 4.0 increased the recovery from less than 10% to about 40% compared with the recovery obtained from a corresponding aqueous solution (Fig. 2). The most likely explanation for this is that the acidic buffer on the recipient side of the dialysis cell changed the pH of the plasma and thereby reduced the degree of protein binding of levosimendan. Because the pK_a value of levosimendan is 6.3 the compound is almost unionized at pH 4.0. Thus the increase in recovery might have resulted from a weaker interaction of neutral levosimendan with HSA compared to that of the anionic form, or from a change in HSA at low pH values resulting in a weaker interaction with the analyte. For example, cortisol, which contains no ionizing group, was released from the corticosteroid-binding globulin and from albumin by lowering the sample pH to 4 (Turnell *et al.*, 1988). A decreased interaction between ketoprofen (pK_a 4.55) and HSA at pH 3, but not at pH 4–7, probably was due to protonation of the anionic compound (Herraéz-Hernández

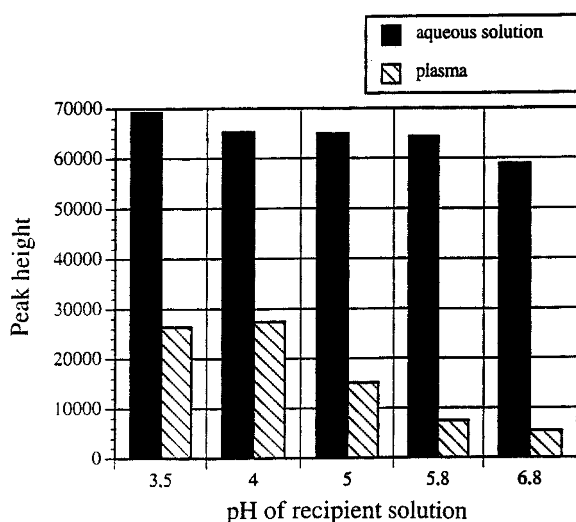

Figure 2. Recovery of levosimendan from human plasma and aqueous solution as a function of pH of recipient solution.

et al., 1995).

Changes in room temperature affected the dialysis rate. An internal standard, differing from the analyte in lacking a methyl group at position 4 in the pyridazinone ring, was thus necessary to improve accuracy.

Carry-over. Between analyses, serious carry-over effects were observed when using 0.86% NaCl as priming solution of the donor side of the dialysis cell. Addition of the detergent Triton X-100 to the priming solution decreased the carry-over remarkably, while addition of small amounts of organic solvents (e.g. methanol) had no effect on it. Use of the same priming solution as for the recipient side of the dialysis cell did not affect the carry-over phenomenon. With a new dialysis membrane the carry-over between analyses was less than 0.1% but it increased with the use of the membrane. In a few cases in which a high-concentration sample was followed by a low-concentration sample, the low-concentration samples had to be re-analysed. The dialysis membrane was replaced after about 500 injections (carry-over $<1\%$). A carry-over effect near 1% may be too high in other types of studies, for instance in toxicological animal studies in which the concentrations of samples can differ by several orders of magnitude.

Trace enrichment

The trace enrichment ODS column delivered with the ASTED system was satisfactory for our purpose. No breakthrough of the analyte was observed under the conditions used. Peak broadening occurred after about 800 analyses and the column was therefore replaced with a new one after about 700 samples.

Validation of the method

Linearity and limit of quantitation. The calibration graphs for plasma samples were linear in the levosimendan concentration range 5–500 ng/mL with correlation coefficients close to 1.000. The limit of detection depends on the volume of plasma in the dialysis cell and the dialysis recovery. Under the conditions used a levosimendan concentration of 5 ng/

Table 2. Within-day precision data for levosimendan in plasma ($n = 6$)

Concentration (ng/mL)	RSD (%)
5	4.0
50	0.8
500	0.7

mL could be detected at a signal-to-noise ratio of 3. The limit of quantitation was the same as the limit of detection because of good precision at that concentration level. In this method a dialysis cell with a 370 μL donor channel was filled with plasma. In applications where the available plasma volumes are not sufficient to fill this volume, 0.9% sodium chloride solution may be used to dilute the sample. Dialysis of 100 μL plasma diluted to 370 μL affords a lower limit of quantitation than dialysis of undiluted plasma by using a commercially available cell with a 100 μL donor channel. We are now investigating the possibility of lowering the limit of quantitation by coupling two dialysis cells in series and injecting larger volumes of plasma or diluted plasma.

Precision and accuracy. The precision and accuracy of the method were evaluated by replicate analysis of spiked plasma samples at different concentrations. The within-day precision and the between-day precision and accuracy of the method are shown in Tables 2 and 3. Based on these results, the method is both accurate and reproducible over the concentration range needed for determinations in clinical studies.

Levosimendan concentrations in plasma samples obtained from clinical studies were determined both with the described ASTED method and with a manual method including liquid–liquid extraction and evaporation. The results corresponded well as shown in Fig. 3. Differences in the results may in part depend on the fact that some samples were stored frozen for several months and thawed more than once between the determinations.

Specificity. Typical chromatograms for plasma analyses are shown in Fig. 4. Chromatograms of unspiked plasma generally do not show any peaks with retention times close to those of levosimendan or the internal standard. No interfering peaks were observed in the chromatograms of samples taken after administration of levosimendan to volunteers. The method is also specific with regard to a large range of drugs used by cardiac failure patients. Of the approximately 30 drugs investigated so far, only carbamazepine gave a peak with a retention time close to that of levosimendan and could therefore interfere with the determination.

Stability of samples. In our ASTED equipment, 64 samples may be loaded into the sample tray, from which the samples are automatically drawn for dialysis. Because the analysis time per sample is 19 min, the time between the first and the

Table 3. Between-day precision and accuracy for levosimendan in plasma ($n = 18$)

Nominal concentration (ng/mL)	Observed concentration (mean ± SD) (ng/mL)	RSD (%)	Error (%)
15	14.7 ± 0.4	2.7	−2.0
150	146 ± 3	2.3	−2.7
450	443 ± 8	1.7	−1.5

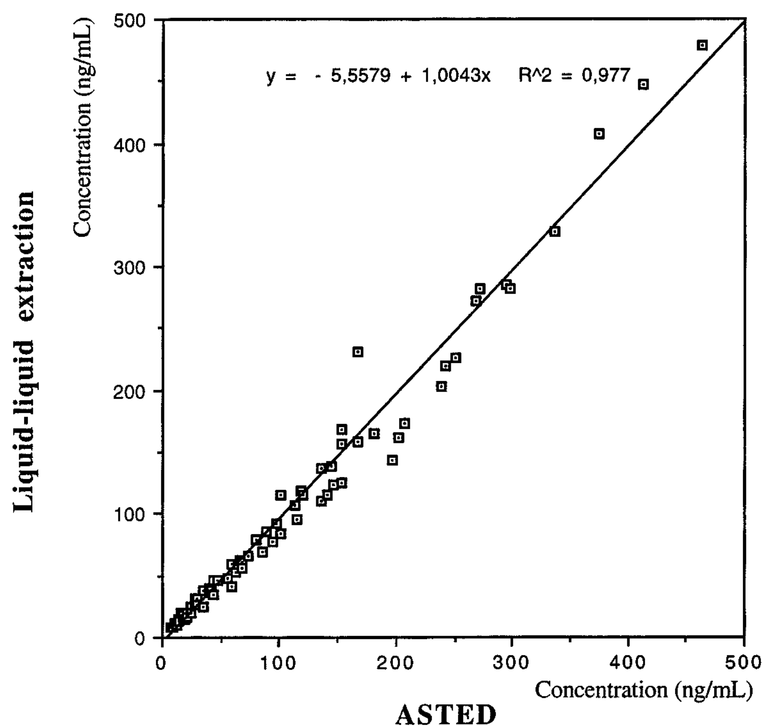

Figure 3. Comparison of ASTED and liquid–liquid extraction sample preparation methods for HPLC analysis of levosimendan (n=73).

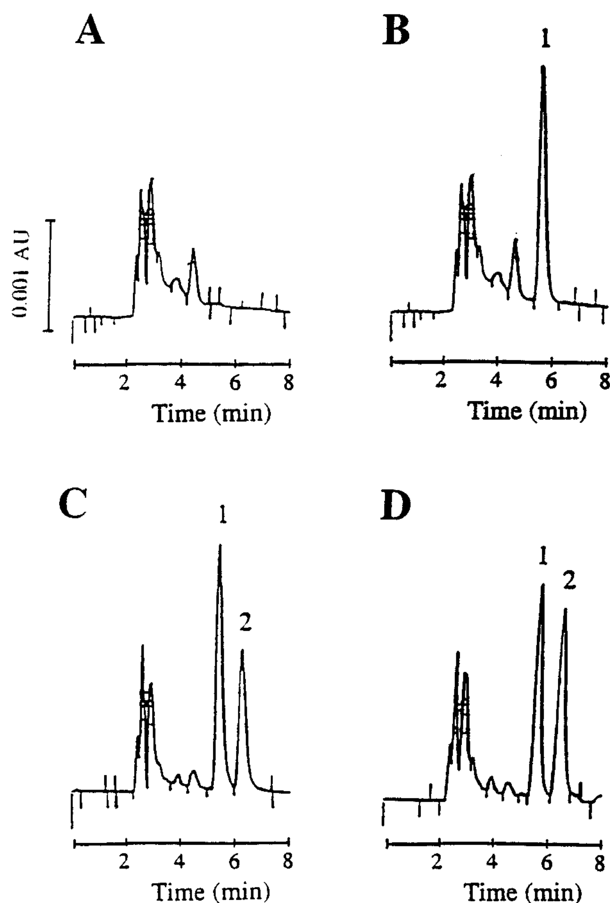

Figure 4. Chromatograms of human plasma samples: (A) blank plasma; (B) plasma with internal standard; (C) 25 ng/mL levosimendan standard; (D) *in vivo* plasma sample. Peak 1, internal standard, retention time 5.4 min; peak 2, levosimendan, retention time 6.4 min.

last analysis may be up to 20 h. Therefore it is of the utmost importance that the analyte is stable in the biological matrix during this time. The stability of levosimendan in human plasma at room temperature was followed for 24 h and no marked changes in the concentrations could be observed. The nominal concentrations were 15 and 150 ng/mL and the determined initial mean concentrations 15 and 147 ng/mL (n=3), while after 24 h the determined mean concentrations were 16 and 145 ng/mL (n=3). The influence of freeze–thaw cycles on the levosimendan concentrations in spiked plasma samples was also studied. The samples were stored at −20 °C and two cycles were studied. No changes in the concentrations were found. When plama samples were stored frozen at −20 °C for 4 months, no marked changes in the concentrations were observed. For longer storage periods, the samples were stored at −70 °C.

CONCLUSIONS

A fully automated HPLC method for quantitation of levosimendan from human plasma was developed. The sample preparation method includes on-line dialysis and trace enrichment of the dialysate. The recovery of this extensively (98%) protein bound drug was greatly improved by lowering the pH of the recipient solution. The method is easy to perform and is thus suitable for routine work. It has been successfully used for the analysis of thousands of plasma samples obtained after both i.v. and p.o. administration of levosimendan with a daily throughput of about 70 samples. With small modifications the method can also be used for the determination of levosimendan from laboratory animal plasma samples.

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